論 文 の 内 容 の 要 旨

論文題目　Roles of RNaseT2 in the Regulation of Macrophage TLR Response to RNA Ligands
（RNAに対するマクロファージTLR応答の制御におけるRNaseT2の役割）

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Introduction

Toll-like receptors (TLRs) are membrane-spanning receptors that play a key role in the innate immune responses. By recognizing pathogen-associated molecular patterns (PAMPs), TLRs induce immune response through activating downstream signaling adaptors, MyD88 and TRIF. The nuclear acid sensing TLRs are localized mainly in endosomal compartments, where they recognize viral and bacterial DNAs and RNAs. TLR3 is a double-stranded RNA (dsRNA) sensor, while TLR7 and TLR8 are single-stranded RNA (ssRNA) sensors. The structures of TLR7 and TLR8 have revealed that they bind to nucleosides and oligoribonucleotides. It has also been reported that TLR8 responds to nucleosides and oligoribonucleotides generated by RNase2 and RNaseT2.

RNases catalyze the cleavage or degradation of various RNAs, including endogenous RNAs, like ribosomal RNA (rRNA) and transfer RNA (tRNA), and pathogen derived RNAs, like viral RNA. Depending on base specificity and optimal pH, RNases are subdivided into three families, RNase A, T1 and T2. In mammals, RNaseT2 is the only member of the RNase T2 family and degrades rRNA and tRNA in endosomes. Mutations in RNaseT2 gene lead to cystic leukoencephalopathy without megalencephaly in humans. This disease was previously studied with RNaseT2-deficient zebrafish, which develops white matter lesions with rRNA accumulation in neurons. Although degradation by RNaseT2 is required for TLR8 responses to ssRNA, the impaired response is not likely to cause inflammatory responses in the brain of RNaseT2-deficient zebrafish.

In this study, I showed the expression of RNaseT2 and its role in the innate immune responses to dsRNA and ssRNA in macrophages. RNaseT2 was highly expressed in macrophages, and its mRNA increased upon stimulation by poly(I:C) or treatment of Type I interferon (IFN). By examination of cytokine mRNA transcription and cytokine production, I found that RNaseT2 negatively regulated TLR3 response to poly(I:C), the dsRNA ligand, whereas it was required for TLR7 response to polyU, the ssRNA ligand. Purified RNaseT2 degraded both the dsRNA, including poly(I:C), and the ssRNA *in vitro*. This indicates RNaseT2's capability of degrading these RNA ligands. Furthermore, with density-gradient centrifugation and confocal microscopy I found that RNaseT2 was localized in the endosomes and lysosomes. With stimulation, RNaseT2 was likely to be transported from early endosomes to late endosomes / lysosomes. RNaseT2 was also found to be colocalized with rhodamine-labeled dsRNA after stimulation. These results strongly suggest that RNaseT2 negatively regulates macrophage TLR3 responses to dsRNA by degrading the ligand, while a prior processing of the ligand by RNaseT2 is required for the TLR7 responses to ssRNA in addition to previously reported TLR8.

Results

1. RNaseT2 was highly expressed in macrophages and its expression increased upon activation.

The expression of RNases including RNase A family and T2 was first investigated in the macrophage cell line J774, and bone marrow derived macrophages (BM-MCs). In both types of cells, RNase4 and RNaseT2 were highly expressed (Fig. 1a-1b). Macrophages in spleen, red pulp macrophages ($F4/80^{+}$ $CD11b^{+}$) and other macrophages ($F4/80^{-}$ $CD11b^{+}$) were also found to expressing RNase4 and RNaseT2. In response to a

variety of ligands including the TLR3 ligand poly(I:C), TLR4/MD-2 ligand Lipid A, STING ligand cGAMP, and IFN-β, expression level of RNase4 was found decreased while that of RNaseT2 increased (Fig. 1c-1d, only BM-MC data are showed, due to similar result in J774 line.). RNaseT2 was also highly expressed in BM-derived plasmacytoid dendritic cells (BM-pDCs) and BM-derived conventional DCs (BM-cDCs). All the results above indicated that RNaseT2 in macrophages may have a role in innate immune responses during bacterial or viral infections. Therefore, I focused on RNaseT2 in further study.

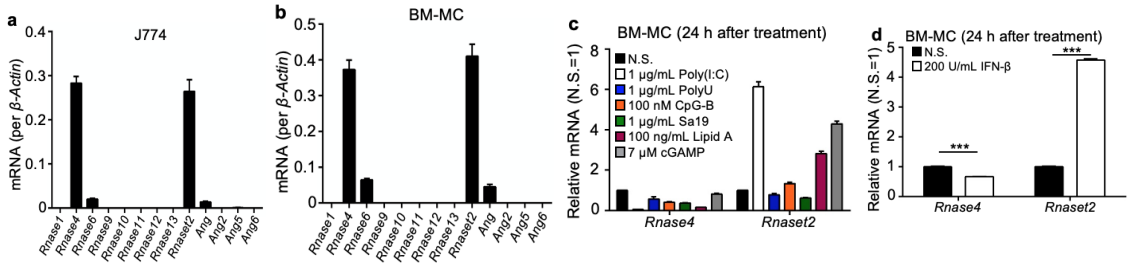

Figure 1. RNase mRNA expressionss in (**a**) J774 macrophage line and (**b**) BM-MCs without stimulation, normalized by *Actb* mRNA. RNase4 and RNaseT2 expression in BM-MCs (**c**) with ligand stimulation and (**d**) with IFN-β treatment, normalized by mRNA in the cells left without stimulation or treatment.

2. RNaseT2 deficiency resulted in TLR3 hyperresponsiveness and TLR7 hyporesponsiveness.

Rnaset2 $^{-/-}$ J774 cell line was established and stimulated with RNA ligands together with the wild-type J774 line, and the expression of CCL5 mRNA as well as CCL5 protein was assesed by real-time PCR or ELISA, respectively. In response to dsRNA ligand poly(I:C) and ssRNA ligand polyU, the CCL5 production increased and decreased, respectively, comparing to that of wild-type cell line (Fig. 2a-2c). *Rnaset2*$^{-/-}$ mice were then constructed to investigate the immune responses in primary immune cells. BM-MCs, BM-cDCs and BM-pDCs were induced from the bone marrow and stimulated with RNA ligands. Similar results to J774 were also earned from BM-MCs (Fig. 2d), BM-cDCs and BM-pDCs. IFN-β mRNA induction by poly(I:C) in J774 line and BM-MCs was also increased by RNaseT2 deficiency. Besides, the TLR7-dependent response to influenza virus in BM-pDC was also abolished by RNaseT2 deficiency. These results strongly suggest that RNaseT2 negatively and positively regulates TLR3 and TLR7 response, respectively.

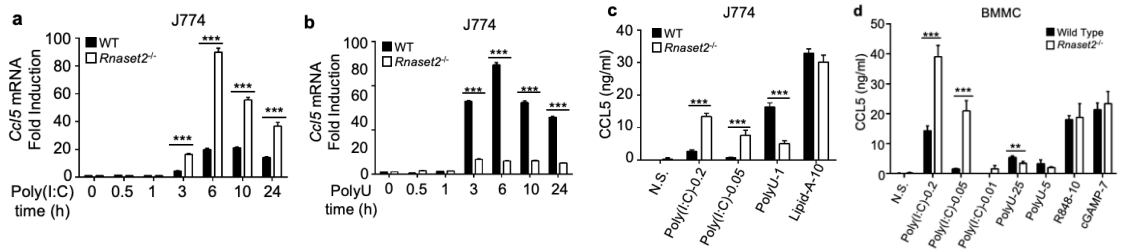

Figure 2. CCL5 induction after ligand stimulation in wild-type and *Rnaset2*$^{-/-}$ cells. *Ccl5* mRNA in J774 cells after indicated time of (**a**) poly(I:C) treatment and (**b**) polyU treatment, normalized by expression level in wild-type cells without stimulation. CCL5 production of (**c**) J774 cells and (**d**) BM-MCs after being stimulated with indicated ligands for 24 hours.

3. RNaseT2 deficiency did not alter TLR expression or RNA uptake.

TLR3 deficiency *Rnaset2*$^{-/-}$ J774 line fully abolished the response to poly(I:C), suggesting that RNase T2 deficiency enhanced TLR3 response in *Rnaset2*$^{-/-}$ J774 cells (Fig. 3a). To investigate the mechanism by which RNase T2 regulates TLR responses, I compared wild-type and RNase T2-deficient cells in the expression of TLR3 and TLR7 by FACS analyses but failed to find any significant change (Fig. 3b, BM-MC data not shown due to similar result to J774). Even though type I IFN treatment increased RNaseT2 mRNA expression (Fig. 1d), TLR3-dependent CCL5 production was enhanced by Type I IFN pretreatment (Fig. 3c), probably because TLR3 expression was also increased with type I IFN treatment (Fig. 3b). These results suggest that RNaseT2 did not alter the expression of TLR3 and TLR7 in its regulation of their responses. To ask whether RNaseT2-deficiency altered RNA uptake, wild-type and *Rnaset2*$^{-/-}$ J774 cell lines were treated with rhodamine-labeled poly(I:C) for up to 30 min before FACS analyses. Since there were no changes in

the brightness of cell fluorescence, poly(I:C) uptake was not enhanced in *Rnaset2*$^{-/-}$ J774 cells (Fig. 3d).

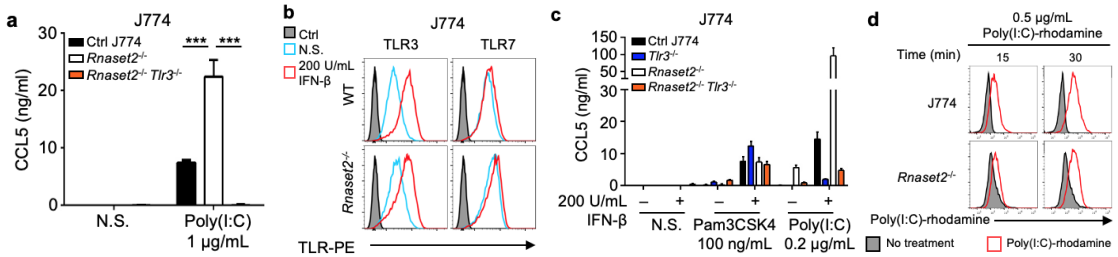

Figure 3. (**a**) CCL5 production after 24 hours of poly(I:C) treatment in indicated J774 cells. (**b**) TLR3 and TLR7 staining in indicated J774 cells with and without IFN-β treatment. (**c**) CCL5 production with and without IFN-β treatment before ligand stimulation. (**d**) Rhodamine fluorescence in J774 cells after poly(I:C)-rhodamine treatment.

4. RNase activity of RNaseT2 was required for the regulation of TLR responses.

To study whether RNase activity is required for the regulation of TLR responses by RNaseT2, three RNaseT2 mutants, i.e., H69A, E118V and H122A, were designed by introducing a mutation into the sites predicted to be required for RNase activity. Another mutant C188R was designed according to the human C184R mutation which causes cystic leukoencephalopathy. The wild-type and mutated RNaseT2-FLAG-His proteins were purified by affinity chromatography, and dsRNA ladder, poly(I:C) or transfer RNA were treated with 50 ng of the purified RNaseT2 proteins at pH 5.0, to mimic the acidic circumstance in lysosomes, before analyses. Degradation of dsRNA ladder, poly(I:C) and transfer RNA was found with wild-type, H69A and E118V RNaseT2, but not with H122A or C188R RNaseT2 (Fig. 4a-4b).

These mutants as well as wild-type RNaseT2 were then transduced into *Rnaset2*$^{-/-}$ J774 cells, and the cells were stimulated with poly(I:C) or polyU, followed by CCL5 production examination. Only the cells transduced with wild-type RNaseT2 or mutants with RNase activity rescued the downregulated poly(I:C) response and upregulated polyU response in *Rnaset2*$^{-/-}$ J774 cells (Fig. 4c-4d). These results suggest that RNA digestion by RNaseT2 negatively regulates TLR3 responses to dsRNA and TLR7 responses require RNA digestion or processing by RNaseT2.

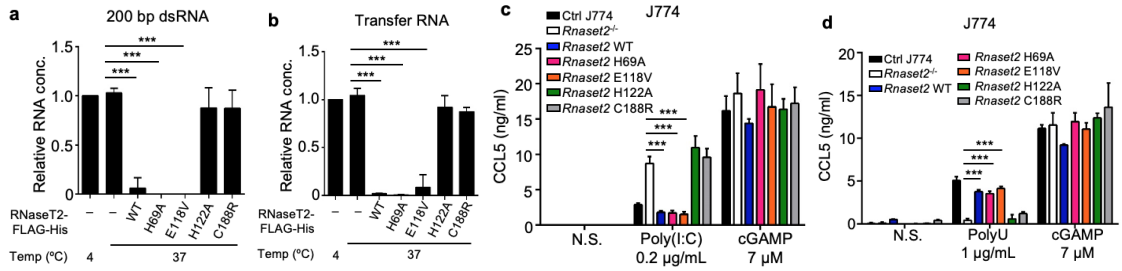

Figure 4. Analyses of (**a**) dsRNA ladder and (**b**) transfer RNA after treatment with purified wild-type and mutated RNaseT2 protein, normalized by the RNA concentration with no RNaseT2 treatment. CCL5 production after (**c**) poly(I:C) and (**d**) polyU stimulation to wild-type and mutated RNaseT2 transduced *Rnaset2*$^{-/-}$ J774 cells.

5. RNaseT2 was localized in late endosomes/lysosomes.

To control TLR3 and TLR7 responses, RNase T2 needs to digest RNAs prior to their interaction with TLR3 and TLR7. The subcellular localization of RNaseT2 was examined first in BM-MCs with density-gradient centrifugation. As a result, RNaseT2 was widely blotted in fractions containing early endosomes, late endosomes, or lysosomes. Interestingly, with an overnight treatment of poly(I:C), RNaseT2 was likely to be transported to late endosomes or lysosomes. As I failed to detect endogenous RNaseT2 under confocal microscopy, J774 cells overexpressing RNaseT2 were stained with anti-RNaseT2 monoclonal antibody as well as antibodies to subcellular organelle markers and then analyzed. Among the organelle markers used in this assay, RNaseT2 showed the highest colocalization with lysosome marker Lamp1, followed by late endosome marker Rab7a (Fig. 5). Colocalization of RNaseT2 and rhodamine-labeled poly(I:C) was also confirmed in the cells pretreated with the labeled ligand (Fig. 5). As TLR3 and TLR7 are also localized in

late endosome or lysosome compartments, these results suggest that RNaseT2 is localized in endosomes and lysosomes to digest incorporated RNA.

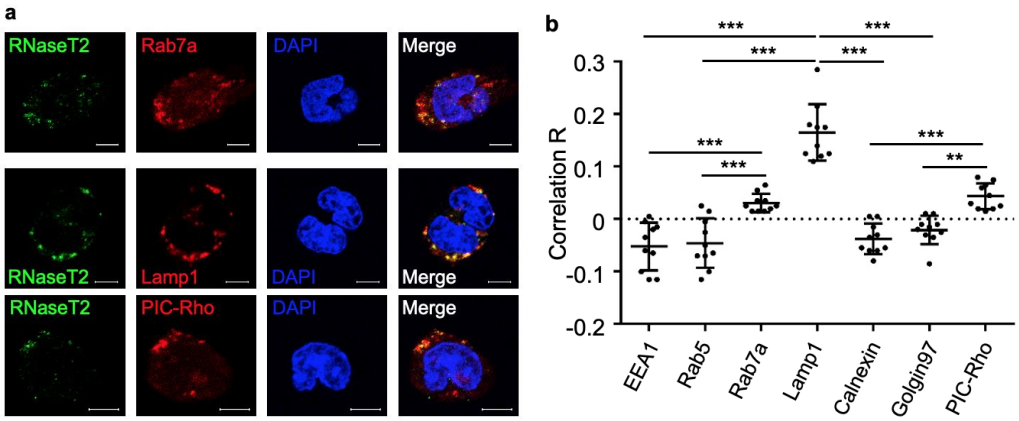

Figure 5. RNaseT2 overexpressing J774 cells analyzed by confocal microscopy. (**a**) Images of cells stained with anti-RNaseT2 mAb and indicated markers or pretreated with rhodamine-conjugated poly(I:C), showing colocalization with these molecules. (**b**) Correlation R of RNaseT2 with oganelle markers or rhodamine-conjugated poly(I:C) calculated from images captured by confocal microscope.

Summary and Discussion

Combining all the results in this study, I found that RNA degradation by RNaseT2 impacts endosomal RNA sensors, including TLR7, the ssRNA sensor and TLR3, the dsRNA sensor in two different directions. (Fig. 6)

Like TLR8 responses, TLR7 responses are likely to depend on prior degradation or processing of RNA into nucleosides and oligoribonucleotides by RNaseT2, as *Rnaset2*$^{-/-}$ J774 cells and BM-MCs showed impaired response to polyU but unaffected response to the small chemical ligand R848. Expression of the loss-of-function mutants of RNaseT2 did not rescue the response to polyU.

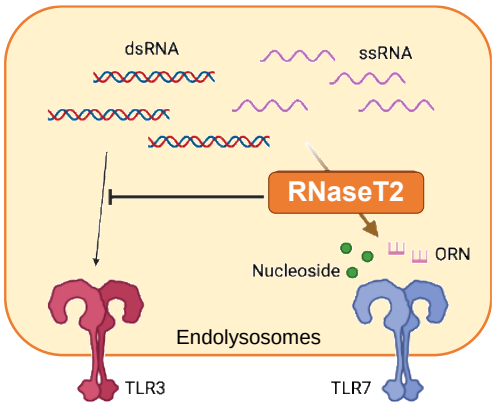

Figure 6. Role of RNaseT2 in RNA responses of TLR3 and TLR7 in late endosomes and lysosomes.

Meanwhile, degradation of dsRNA including poly(I:C) by RNaseT2, as is presented in my study, negatively regulates TLR3 response. Although dsRNA is considered resistant to RNase digestion, purified RNaseT2 degraded commercial dsRNA ladder as well as poly(I:C) *in vitro*, and H122 was identified as an essential site for the RNase activity. Through density-gradient centrifugation and confocal microscopy, RNaseT2 was found to be localized in endosomes and lysosomes, and colocalization of RNaseT2 and labeled dsRNA was confirmed in J774 cells, which suggests that late endosome and lysosome are the places where RNA is degraded by RNaseT2.

RNaseT2 mRNAs increased after Type I IFN treatment. RNaseT2 might have a role in defense responses against viruses when Type-I IFNs are produced. In this context, TLR7 responses might be upregulated by increased RNaseT2 expression. Interestingly, TLR3 responses were also enhanced by Type I IFN treatment despite increased expression of the negative regulator RNaseT2, which might be explained by increased TLR3 expression in Type I IFN-treated macrophages. These results suggest that Type I IFNs increase macrophage responses to RNA by increasing TLR3, an RNA sensor itself, and RNaseT2, an enzyme generating ligands for another RNA sensor.

RNA digestion by RNaseT2 had opposite impacts on TLR3 and TLR7. Tissue-resident macrophages such as red pulp macrophages in the spleen, microglia in the brain, alveolar macrophages in the lung, and cardiac macrophages express both TLR3 and TLR7. In these macrophages, RNaseT2 is likely to skew the balance of endosomal responses to RNAs, from TLR3 to TLR7.